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International Bu

WO 9608213A1

INTERNATIONAL APPLICATION PUBLISHED UNDER

(51) International Patent Classification 6 :
A61F 2/24

A1

(11) International Publication Number: WO 96/08213

(43) International Publication Date: 21 March 1996 (21.03.96)

(21) International Application Number: PCT/US95/11395

(22) International Filing Date: 8 September 1995 (08.09.95)

(30) Priority Data:
304,062 12 September 1994 (12.09.94) US
488,165 7 June 1995 (07.06.95) US

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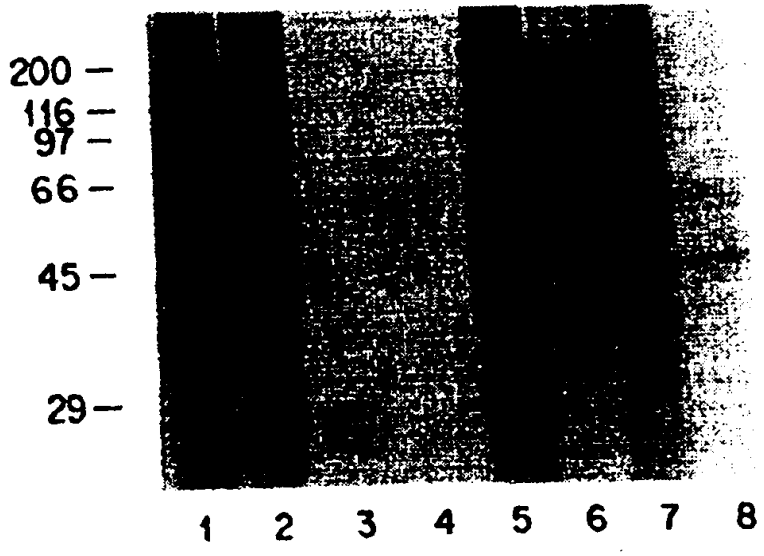
(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TM, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.

(54) Title: THREE-DIMENSIONAL HUMAN CELL CULTURES ON CARDIAC VALVE FRAMEWORKS AND THEIR USES

(57) Abstract

The present invention relates to a method of growing a variety of different cells and tissues in three-dimensional cultures *in vitro* using human fibroblasts in the culture medium. In accordance with the invention, stromal cells, including but not limited to human dermal and cardiac fibroblasts, are inoculated and grown on a three-dimensional scaffold or framework. The human fibroblasts secrete human matrix proteins to supplement and replace the existing porcine matrix composed of decellularized heart valves or aortic walls and leaflets formed into three-dimensional constructs having interstitial spaces bridged by the stromal cells. The living stromal tissue so formed provides the support, growth factors, and regulatory factors necessary to sustain long-term viability and proliferation of cells in culture and/or cultures implanted *in vivo*. When grown in this three-dimensional system, the proliferating cells mature and segregate properly to form components of tissues analogous to counterparts *in vivo*.



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THREE-DIMENSIONAL HUMAN CELL CULTURES ON CARDIAC VALVE FRAMEWORKS AND THEIR USES

1. INTRODUCTION

The invention relates to growing in vitro, human cells such as fibroblasts on a three-dimensional scaffold, comprising porcine aortic leaflets and walls, intact heart valves, other biological scaffolding suitable for reconstructing a valve or valve components, for example, including but not limited to the pericardium or the small intestinal submucosa, and biodegradable frameworks, such that the scaffold is populated with viable human cells having normal function, and fibroblasts are stimulated to produce human matrix proteins to supplement and replace the existing matrix on the scaffold.

The resulting three-dimensional tissue constructs have a variety of applications ranging from transplantation or implantation in vivo for replacement and/or reconstruction of a single valve component or the entire heart valve, to screening cytotoxic compounds and pharmaceutical compounds in vitro.

2. BACKGROUND OF THE INVENTION

Valve replacement surgical therapy is required for the treatment of various types of valvular heart diseases, including, but not limited to, aortic stenosis, aortic regurgitation, mitral stenosis, mitral regurgitation, pulmonary valve disease, tricuspid valve disease, multivalvular disease, Marfan syndrome and artificial valve disease. Two general types of valve replacement are available: the artificial, mechanical prosthesis or valve, and tissue biological prosthesis or valve. There are several kinds of mechanical prosthesis, such as the ball valve, the tilting disk and the central flow disk. There are also several tissue prostheses,

including preserved homografts and stent-mounted, porcine valve heterografts.

The primary advantage of the mechanical prosthesis is durability, whereas the disadvantage is a requirement that patients be on an anticoagulant therapy to reduce the risk of thromboembolic complications. This is because artificial mechanical heart valves are prone to occlusions by thrombus, and are subject to mechanical failures. Thromboembolism and anticoagulated hemorrhage are still the frequent causes for reoperation and patient death. Moreover, mechanical failure can occur suddenly and without warning resulting in emergency surgical interventions for replacement of the device. The advantage of the biological prothesis is a lower risk of thromboembolic complications, but may still require anticoagulant therapy in some situations. Moreover, the biological grafts which are currently used are not prone to sudden failure. However, the biological tissue grafts are also limited for a number of reasons. Allogeneic human valves (derived from a donor of the same species) are limited by the supply of donated human hearts and recipients must undergo immunosuppressive therapy to avoid rejection. While xenogeneic valves (derived from a donor of a different species) are more plentiful, they require treatment that results in calcification and a gradual degradation over time, requiring replacement. For example, allogeneic, transplanted heart valves may be obtained fresh, or may be cryopreserved to maintain viability of cellular components. Patients receiving allogeneic transplants usually must undergo immunosuppressive therapy. Despite such therapy, many of the transplants become inflamed and fail within five to ten years. Moreover, allogeneic valves are not as readily available as the xenogeneic valves. Xenogeneic biological valves, usually porcine or bovine in origin, have the advantage of being identical in design and structure to those valves being replaced, but are fixed

with glutaraldehyde and, therefore, are non-living. The glutaraldehyde-treated tissues calcify over time and do not allow infiltration and colonization by host cells, which is necessary for remodeling. Consequently, these xenogeneic valves degrade with time and eventually malfunction.

Autologous human tissue (i.e., derived from the recipient) is used for coronary and peripheral bypass procedures, third degree burns and reconstructive procedures involving bones and cartilage grafting. Such use eliminates complications of immunorejection resulting in better graft survival. Unfortunately, complications ensue with autologous heart valve transplants e.g., thrombosis and occlusion in the post-implant period and scarring of implant tissue. The development of alternative, tissue-based heart valves for transplantation is necessary due to unmet patient demands to improve upon existing heart valve technologies, which are mechanical valves requiring the constant use of anticoagulants and glutaraldehyde fixed tissue valves which eventually experience calcification.

Previous attempts at producing artificial tissues and organs have met limited success. Orton, U.S. Patent No. 5,192,312 describes treating a transplant tissue sample with exogenous basic fibroblast growth factor (BFGF) and repopulating the tissue with cells, preferably allogeneic or autogenous fibroblasts, ostensibly to avoid immunological rejection. The heart valves and heart leaflets may be sterilized with lethally effective doses of x-rays or with antibiotics, antibacterials and cytotoxic agents. According to Orton, the addition of bFGF to the tissue in vitro is critical in this system, and is essential for causing the graft-populating cells to migrate into the tissue and proliferate in response to the growth factor, and populate the tissue. However, Orton only demonstrates the system on small pieces of the valve fixed in a petri dish and does not show production

of a functional heart valve or any of the alleged advantages if implanted in vivo, e.g., avoidance of immunological rejection, and reduction in hardening and/or scarring of the valve transplant.

Livesey et al., U.S. Patent No. 5,336,616 relates to a method of producing a transplantable tissue graft for processing and preserving acellular, collagen-based tissue matrix for transplantation. The method described involved processing biological tissues with a stabilizing solution to prevent osmotic, hypoxic, autolytic and proteolytic degradation and to control contamination. The tissues were decellularized with EDTA, CHAPS or a zwitterionic detergent, SDS or anionic/nonionic detergent, followed by treatment with a cryoprotectant such as DMSO, propylene glycol, butanediol, raffinose, polyvinyl pyrrolidone, dextran or sucrose and vitrified in liquid nitrogen. Thereafter, the tissues were subjected to a dry stabilization procedure involving molecular distillation drying under nitrogen gas, followed by rehydration with buffered solution. Each of the methods has limitations and therefore it is essential that very stringent measures be taken to preserve the biological properties of the material and avoid toxicity resulting from reagents used during processing.

3. SUMMARY OF THE INVENTION

The present invention relates to transplantable cardiac tissue or bioprosthetic grafts composed of human cells grown on three-dimensional frameworks, scaffolds or matrices, a method of culturing human cells on such frameworks and uses of such three-dimensional cell cultures. In accordance with the invention, stromal cells, including but not limited to human fibroblasts, are inoculated and grown on a three-dimensional frameworks, such as intact heart valves, aortic walls and leaflets, or other biological scaffolding suitable for reconstructing a valve or valve components, including for

example, but not limited to the pericardium or the small intestinal submucosa or biodegradable frameworks or matrices. The preferred three-dimensional framework may be prepared from intact porcine heart valves, aortic wall tissue, or leaflets which are decellularized (at -20°C to -70°C or with detergents and enzymes) and sterilized by: chemical methods including, but not limited to, ethylene oxide and peracetic acid; irradiation including, but not limited to, gamma and electron beam; and steam sterilization including, but not limited to autoclaving. No viable cells remain in the decellularized/sterilized tissue samples which are used as a scaffold or framework for culturing the stromal cells.

The stromal cells which are inoculated onto the scaffold, may include dermal or cardiac fibroblasts, and/or cells capable of producing collagen types I and III, and in some instances, elastin, which are typically produced in heart valves. (See Table I). The stromal cells and connective tissue proteins naturally secreted by the stromal cells attach to and substantially envelope the three-dimensional framework or construct, having interstitial spaces bridged by the stromal cells. The living stromal tissue so formed provides the support, growth factors, and regulatory factors necessary to sustain long-term active proliferation of stromal cells in culture and/or cultures implanted in vivo. When grown in this three-dimensional system, the proliferating cells mature and segregate properly to form components of adult tissues analogous to counterparts in vivo.

In another embodiment of the invention, the stromal cells can be genetically engineered to express a gene product beneficial for successful and/or improved transplantation. For example, the stromal cells can be genetically engineered to express anticoagulation gene products to reduce the risk of thromboembolism, or anti-inflammatory gene products to reduce the risk of failure due to inflammatory reactions. For example, the stromal

cells can be genetically engineered to express tissue plasminogen activator (TPA), streptokinase or urokinase to reduce the risk of clotting. Alternatively, the stromal cells can be engineered to express anti-inflammatory gene products, e.g., peptides or polypeptides corresponding to the idiotype of neutralizing antibodies for tumor necrosis factor (TNF), interleukin-2 (IL-2), or other inflammatory cytokines. Preferably, the cells are engineered to express such gene products transiently and/or under inducible control during the post-operative recovery period, or as a chimeric fusion protein anchored to the stromal cell, e.g., a chimeric molecule composed of an intracellular and/or transmembrane domain of a receptor or receptor-like molecule, fused to the gene product as the extracellular domain.

In another alternative, the stromal cells can be genetically engineered to "knock out" expression of factors or surface antigens that promote clotting or rejection. For example, expression of fibrinogen, von Willebrands factor or any cell surface molecule that binds to the platelet $\alpha 2B\beta$-3 receptor can be knocked out in the stromal cells to reduce the risk of clot formation. Likewise, the expression of MHC class II molecules can be knocked out in order to reduce the risk of rejection of the graft.

In yet another embodiment of the invention, the three-dimensional culture system of the invention may afford a vehicle for introducing genes and gene products in vivo to assist or improve the results of the transplantation and/or for use in gene therapies. For example, genes that prevent or ameliorate symptoms of valvular disease such as thrombus formation, inflammatory reactions, fibrosis and calcification, may be underexpressed or overexpressed in disease conditions. Thus, the level of gene activity in the patient may be increased or decreased, respectively, by gene replacement

therapy by adjusting the level of the active gene product in genetically engineered stromal cells.

In a specific embodiment exemplified by the examples in Section 6, infra, human dermal fibroblasts were grown in the three-dimensional culture systems of the invention. Porcine aortic walls and leaflets were chosen because they are currently used in replacement therapy of heart valves. Particular benefits were achieved in porcine aortic wall and leaflet cultures where proliferation of human fibroblasts occurred, and production of tissue similar to human matrix proteins in the aortic walls and leaflets was detected. These characteristics were monitored by analyzing the recellularized or remodeled constructs for cell distribution (histological analyses), cell viability (MTT assays), cell proliferation (^{3}H-Thymidine labeling or BrdU incorporation), protein production (^{3}H-proline labeling, ^{35}S-cysteine/methionine labeling), and protein immunohistochemistry. The results from these studies showed that human dermal fibroblasts were able to colonize the porcine scaffolding of leaflets, aortic wall biopsies and intact valves, cultured and grown over several time intervals, for example, but not limited to 2, 4, 8 and 18 weeks. The present invention, thus, relates to a method of repopulating porcine aortic walls and leaflets with human fibroblasts to produce human matrix proteins in which the porcine aortic leaflets and walls are first sterilized with peracetic acid (or by other chemical means such as ethylene oxide) or by radiation with an electron beam (or by gamma irradiation) or by steam (autoclaving).

In the examples described infra, human fibroblasts were grown in culture on frameworks or constructs, composed of porcine aortic valves, walls and leaflets which had been decellularized and sterilized. When implanted in vivo, such frameworks or constructs allow adequate nutrient and gas exchange to the cells until

engraftment and vascularization at the site of engraftment occurs. The advantage of adding human fibroblasts to the three-dimensional, decellularized porcine scaffolds or biodegradable constructs, is that colonization of the porcine scaffolding results in a valve implant with living cells which produce biological factors that may stimulate host cells to endothelize the implant and stimulate host cardiac fibroblasts to integrate into the implant. The net result is enhancement of host-graft take. Another advantage of adding human fibroblasts is that cultures can be maintained under sterile conditions without inhibiting the growth of human fibroblasts, which grow in various types of frameworks or constructs usually pretreated with detergents or enzymes and sterilized with peracetic acid or irradiation with the electron beam. Furthermore, heart valves colonized with functional human cells are less likely to be subject to immunological rejection and thus are superior to those heart valves which are covered with xenogeneic cells prepared for use in replacement therapy.

It is an object of the present invention to construct a heart valve from human foreskin or cardiac fibroblasts and porcine heart valve and/or aortic walls and leaflets, which no longer contains porcine cells but becomes a humanized porcine heart valve or a recellularized heart valve suitable for transplantation in humans. Such an approach provides an improved method and means of designing, constructing and utilizing aortic walls and leaflets, intact heart valves other biological scaffolding suitable for reconstructing a valve or valve component (e.g., pericardium, small intestinal submucosa, etc.) and biodegradable frameworks, as scaffolding for growth and implantation of human fibroblasts in vitro.

It is further the object of the invention to construct a heart valve consisting of human cells and human tissue matrix proteins made by human dermal or

cardiac fibroblasts and a completely or nearly complete bioresorbable/biocompatible polymer scaffolding in the shape of different types of valves or their components, for example, but not limited to aortic, pulmonary, mitral, and tricuspid valves. Such an approach provides bioprosthetic or transplantable tissues, which can be utilized for cell growth, both in vitro and in vivo, to replace or reconstruct degenerated and dysfunctional heart valves in human patients.

It is a still further object of this invention to use human dermal or cardiac fibroblasts to colonize the porcine aortic leaflets and wall biopsies or other biological scaffolding suitable for reconstructing a valve or valve components (e.g., pericardium, small intestinal submucosa, etc.), and remain metabolically viable with the result that all porcine cells native to the leaflet and wall tissues or other biological scaffolds, are either eliminated (decellularized) or nonviable (dead). Such an approach provides an in vitro system in which human fibroblast cells retain their morphology and cell function for the secretion of bioactive molecules normally produced in the body by the cells of the aortic walls and leaflets or the intact heart valve or the pulmonary, mitral, and tricuspid valves.

The present invention relates to methods and biological tissue prothesis or valves for the treatment of valvular heart disease, including, but not limited to, aortic stenosis, aortic regurgitation, mitral stenosis, mitral regurgitation, pulmonary valve disease, tricuspid valve disease, multivalvular disease, tricuspid valve disease, Marfan syndrome and artificial valve disease.

4. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a photograph of autoradiographed proteins synthesized by human dermal fibroblasts post seeding onto porcine aortic leaflets and walls.

Figure 2 is a photograph of hematoxylin and eosin stained tissue sections: a) a fresh porcine leaflet (the cardiac fibroblast nuclei native to the tissue appear purple in coloration); b) a detergent and/or enzyme extracted porcine leaflet (no porcine cell nuclei are detected after chemical treatment); c) a detergent and/or enzyme extracted porcine leaflet cultured with human fibroblasts for 18 weeks (the dermal human fibroblasts are present in the porcine matrix). (Stained with Hematoxylin/Eosin.) (10x).

Figure 3 is a photograph of a porcine leaflet seeded with human dermal fibroblasts and cultured for 4 weeks. (Stained with Hematoxylin/Eosin.)

Figure 4 is a bar graph showing that in three sample sets (#1-3) of detergent and/or enzyme extracted leaflets with or without fibroblasts, only the leaflets which are grown with fibroblasts incorporated ^{3}H-thymidine, indicating that the fibroblasts were proliferating.

Figure 5 is a SDS gel autoradiograph analysis showing protein bands: non-viable porcine leaflet (lane 1) and wall biopsy (lane 2) seeded with human fibroblasts show protein synthesis, whereas unseeded, non-viable porcine leaflet (lane 3) and porcine wall biopsy (lane 4) show no activity. Fresh, viable porcine leaflet (lane 5) and wall biopsy (lane 6) seeded with human fibroblasts have similar patterns to fresh, viable, unseeded porcine leaflet (lane 7) and wall biopsy (lane 8).

Figure 6. Porcine leaflet and wall (negative controls) a,b, respectively) stained with serum only and showed no background staining. Porcine leaflet stained with human tenascin (c) and porcine wall stained with human fibroblast antibody (d). Both c and d show no species cross reactivity. Whole humanized porcine valve

constructs cultured for 4 weeks under dynamic flow showed positive staining for human tenascin in the leaflet, wall, and muscle bar (e,g,i) and positive staining for human fibroblasts in the leaflet, wall, and muscle bar (f,h,j).

Figure 7 is a photograph of autoradiographed protein incorporation of human fibroblasts after dynamic culture on porcine aortic leaflets.

Figure 8 is a photograph depicting human fibroblast proliferation on a porcine matrix which was previously decellularized by detergent and/or enzyme treatment. The proliferating cells were labeled with Brdu and detected using an antibody to Brdu and a visualization kit. The labeled cells proliferating on the tissues were grown under dynamic flow conditions. Brdu labeling occurred during the last 72 hr of a 4 week culture period.

Figure 9 is a photograph depicting decellularized (detergent and/or enzyme) + electron beamed valves seeded with human fibroblasts under pulstile (left) and non-pulstile (right) dynamic flow conditions. Both valves were cultured dynamically for 1 week, then stained with MTT. The valve seeded with pulstile flow conditions had greater and more uniform fibroblast attachment.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to transplantable cardiac tissue constructs or bioprosthetic grafts grown in three-dimensional frameworks, a method of culturing human cells on such frameworks and uses of such three-dimensional, recellularized tissue constructs grown in cultures. In accordance with the invention, stromal cells, including but not limited to human fibroblasts, are inoculated and grown on a three-dimensional framework or construct of intact heart valves, aortic walls and

leaflets or other biological scaffolding suitable for reconstructing a valve or valve components, for example, including but not limited to the pericardium or the small intestinal submucosa or biodegradable frameworks. Cells grown on a three-dimensional framework, in accordance with the present invention, grow to form a cellular tissue-matrix which resembles tissue found in vivo to a greater degree than previously described. The three-dimensional cell culture system treated with human stromal cells is applicable to the proliferation of different types of cells and formation of a number of different tissues, including but not limited to aortic walls and leaflets, or intact heart valves, pulmonary, mitral, and tricuspid valves. In addition, the stromal cells grown in the system may be genetically engineered to produce gene products beneficial to transplantation, e.g., anti-coagulation factors, e.g., TPA, streptokinase, etc., or anti-inflammatory factors, e.g., anti-TNF, anti-IL-2, etc. Alternatively, the stromal cells may be genetically engineered to "knock out" expression of native gene products that promote platelet binding and clot formation, e.g., fibrinogen, von Willebrands factor, or "knock out" expression of MHC in order to lower the risk of rejection. In addition, the stromal cells may be genetically engineered for use in gene therapy to adjust the level of gene activity in a patient to assist or improve the results of the transplantation.

The use of human foreskin fibroblasts in the three-dimensional tissue constructs has a variety of advantages and applications. For example, for a variety of cells and tissues, such as porcine heart valves, aortic walls and leaflets, the chordae tendinea in the mitral and tricuspid valve, skin, ligaments, tendons, etc., the three-dimensional tissue constructs can be produced at a rapid rate and may itself be transplanted or implanted into a living organism without undue delay. The three-dimensional tissue constructs may also be used in vitro

for testing the effectiveness or cytotoxicity of pharmaceutical agents, screening compounds for use in treatment of clotting or thromboembolism, as anticoagulants, as anti-inflammatory agents, as anti-calcification agents or as endothelialization agents.

In yet another application, the three-dimensional tissue construct system may be cellularized within a "bioreactor" to produce a valve or valve component with leaflet mobility and full valve function. For example, an intact valve comprising of leaflets attached to the wall, may be assembled as a three-dimensional framework, inoculated with human stromal cells and maintained in recirculating culture medium regulated by a peristaltic or pneumatic pump which also keeps the leaflets or tissue sheets/patches in a dynamic state. The bioreactor provides a closed system free from problems of contamination during procedures involving sterilization, seeding, culturing, shipping and/or testing valve function.

The methods for culturing cells including human dermal fibroblasts on aortic walls and leaflet cells or intact heart valves or other biological scaffolding suitable for reconstructing a valve or valve components, for example, but not limited to the pericardium or the small intestinal submucosa or biodegradable frameworks, as a three-dimensional biological or synthetic framework or construct which can be used in accordance with the invention are described in applicants' co-pending application Serial No. 08/304,062 filed September 12, 1994; which is a continuation-in-part of Serial No. 08/254,096 filed June 6, 1994; which is a continuation-in-part of Serial No. 08/131,361 filed October 4, 1993, U.S. Patent No. 5,041,138 by Vacanti et al., and application Serial No. 07/509,952 filed April 16, 1990 by Vacanti et al., each of which is incorporated by reference herein in its entirety.

Methods for the treatment of valvular heart disease, including, but not limited to, aortic stenosis, aortic regurgitation, mitral stenosis, mitral regurgitation, pulmonary valve disease, tricuspid valve disease, multivalvular disease, tricuspid valve disease, Marfan syndrome and artificial valve disease, are described.

5.1. ESTABLISHMENT OF THREE-DIMENSIONAL FRAMEWORK

The three-dimensional framework for use in the present invention may be of any material and/or shape that: (a) allows cells to attach to it (or can be modified to allow cells to attach to it); and (b) allows cells to grow in more than one layer. It is preferred that allogeneic and xenogeneic aortic walls and leaflets or intact heart valves or other biological scaffolding suitable for reconstructing a valve or valve components, for example, but not limited to the pericardium or the small intestinal submucosa or biodegradable frameworks, obtained from a variety of mammals, including but not limited to, man, pig, cow, sheep or dog, may be used. The porcine leaflets and aortic biopsies may be used in the following forms: irradiated or chemically treated or steam treated (sterilized); decellularized (for example, detergent and/or enzyme treated), extracted and sterilized; and valve tissue with nonviable cells and other biological tissues, for example, but not limited to, pericardium or small intestinal submucosa (accomplished by such procedures as freezing at -20°C to -70°C, or by repeated freezing and thawing).

The methods for decellularizing the aortic walls and leaflets or intact valves or other biological scaffolding suitable for reconstructing a valve or valve components, which can be used in accordance with the invention, include, but are not limited to the methods described in U.S. Patent No. 5,336,616 and U.S. Patent No. 4,776,853, which are incorporated herein by reference in their

entirety. For example, the tissues can be decellularized with EDTA, CHAPS or a zwitterionic detergent, followed by treatment with a cryoprotectant such as DMSO, propylene glycol, butanediol, raffinose, polyvinyl pyrrolidone, dextran or sucrose and vitrified in liquid nitrogen. Alternatively, the tissue sample can be subjected to enzymatic digestion and/or extracting with reagents that break down the cellular membranes and allow removal of cell contents. Examples of detergents include non-ionic detergents (for example, TRITON X-100, octylphenoxy polyethoxyethanol, (Rohm and Haas); BRIJ-35, a polyethoxyethanol lauryl ether (Atlas Chemical Co.), TWEEN 20, a polyethoxyethanol sorbitan monolaureate (Rohm and Haas), LUBROL-PX, or polyethylene lauryl ether (Rohm and Haas)); and ionic detergents (for example, sodium dodecyl sulphate, sulfated higher aliphatic alcohol, sulfonated alkane and sulfonated alkylarene containing 7 to 22 carbon atoms in a branched or unbranched chain). The enzymes used may include nucleases (for example, deoxyribonuclease and ribonuclease), proteases, phospholipases and lipases. The tissues in the invention can also be decellularized using physical procedures such as ultrasonic treatment or osmotic shock, or by chemical treatment using peracetic acid.

The three-dimensional framework may also be composed of completely or nearly complete bioresorbable/ biocompatible polymer scaffolding in the shape of various different types of valves, including but not limited to, aortic, pulmonary, mitral, and tricuspid valves and valve components of each type. The biodegradable scaffolds, constructs, frameworks or matrices may be composed of materials such as polyglycolic acid, catgut suture material, hyaluronic acid, cellulose, collagen (in the form of sponges, braids, or woven threads, etc.), gelatin, or other naturally occurring biodegradable materials or synthetic materials, including for example, a variety of polyhydroxyalkanoates. Such frameworks or

constructs may be molded into the shape of heart valves or repair sheets/patches prior to inoculation of human cells. Where possible, however, it is most preferable to use a three-dimensional construct of the tissue of origin, for example, the aortic walls and leaflets or intact heart valves.

The invention is based in part, on the discovery that the three-dimensional system supports the proliferation, migration, differentiation, and segregation of cells in culture in vitro to form components of tissues analogous to counterparts found in vivo. The human cells added to the scaffolds repopulate the porcine valve without the need for exogenously added growth factors. This is contrary to Orton's teachings which show that leaflet tissue not treated with bFGF remained acellular. The use of growth factors (for example, but not limited to, αFGF, βFGF, insulin growth factor or TGF-betas), or natural or modified blood products or other bioactive biological molecules (for example, but not limited to, hyaluronic acid or hormones), even though not absolutely necessary in the present invention, may be used to further enhance the reconstitution of the porcine or other biological scaffolding.

Although the applicants are under no duty or obligation to explain the mechanism by which the invention works, a number of factors inherent in the three-dimensional culture system may contribute to its success:

(a) The three-dimensional framework provides a greater surface area for protein attachment, and consequently, for the adherence of stromal cells.

(b) Because of the three-dimensionality of the framework, stromal cells continue to actively grow, in contrast to many cells in monolayer cultures, which grow to confluence, exhibit contact inhibition, and cease to grow and divide. The elaboration of extracellular matrix

proteins and secretion of growth and regulatory factors by replicating stromal cells may be partially responsible for stimulating proliferation, maintaining normal tissue differentiation and regulating differentiation of cells in culture.

(c) The three-dimensional framework allows for a spatial distribution of cellular elements which is more analogous to that found in the counterpart tissue in vivo.

(d) The increase in potential volume for cell growth in the three-dimensional system may allow the establishment of localized microenvironments conducive to cellular maturation.

(e) The three-dimensional framework maximizes cell-cell interactions by allowing greater potential for movement of migratory cells.

(f) It has been recognized that maintenance of a differentiated cellular phenotype requires not only growth/differentiation factors but also the appropriate cellular interactions. The present invention effectively recreates the tissue microenvironment.

The three-dimensional stromal support, the culture system itself, and its maintenance, as well as various uses of the three-dimensional cultures are described in greater detail in the subsections below.

5.2. ESTABLISHMENT OF THREE-DIMENSIONAL STROMAL TISSUE

Stromal cells comprising fibroblasts, with or without other stromal cells and elements described below, are inoculated onto the three-dimensional framework. Human fibroblasts may be added to the culture prior to, during or subsequent to inoculation of other stromal cells. The concentration of fibroblasts maintained in the cultures can be monitored and adjusted appropriately to optimize growth and to regulate scaffold colonization. Alternatively, stromal cells that are genetically

engineered to express and produce factors similar to those produced by cells of the heart valve, may be included in the inoculum. These cells could serve as a source of protein factor(s) in the culture. Preferably, the gene or coding sequence for factor(s) would be placed under the control of a regulated promoter, so that production of factor(s) in culture can be controlled. The genetically engineered cells will be screened to select those cell types: 1) that bring about amelioration of blood clotting, coagulation, thromboembolism and inflammatory reactions in vivo, and 2) escape immunological surveillance and rejection.

Stromal tissue comprising dermal fibroblasts, cardiac fibroblasts and cells capable of producing collagen type I and III, elastin and other heart valve matrix proteins, for example, but not limited to fibronectin and glycosaminoglycans, are used to grow in vitro, transplantable tissue or bioprosthetic heart valves. Stromal cells such as fibroblasts can be obtained in quantity rather conveniently from skin, human foreskin, heart tissue or any appropriate organ. Fetal and neonatal fibroblasts can be used to form a "generic" three-dimensional stromal tissue construct that will support the growth of a variety of different cells and/or tissues. Fibroblasts may be readily isolated by disaggregating an appropriate organ or tissue which is to serve as the source of the fibroblasts. This may be readily accomplished using techniques known to those skilled in the art. For example, the tissue or organ can be disaggregated mechanically and/or treated with digestive enzymes and/or chelating agents that weaken the connections between neighboring cells making it possible to disperse the tissue into a suspension of individual cells without appreciable cell breakage. Enzymatic dissociation can be accomplished by mincing the tissue and treating the minced tissue with any of a number of digestive enzymes either alone or in combination. These

include but are not limited to trypsin, chymotrypsin, collagenase, elastase, hyaluronidase, pronase, etc. Mechanical disruption can also be accomplished by a number of methods including, but not limited to the use of grinders, blenders, sieves, homogenizers, or pressure cells to name but a few. For a review of tissue disaggregation techniques, see Freshney, Culture of Animal Cells. A Manual of Basic Technique, 2d Ed., A.R. Liss, Inc., New York, 1987, Ch. 9, pp. 107-126.

Once the tissue has been reduced to a suspension of individual cells, the suspension can be fractionated into subpopulations from which the fibroblasts and/or other stromal cells and/or elements can be obtained. This also may be accomplished using standard techniques for cell separation including but not limited to cloning and selection of specific cell types, selective destruction of unwanted cells (negative selection), separation based upon differential cell agglutinability in the mixed population, freeze-thaw procedures, differential adherence properties of the cells in the mixed population, filtration, conventional and zonal centrifugation, centrifugal elutriation (counter-streaming centrifugation), unit gravity separation, counter current distribution, electrophoresis and fluorescence-activated cell sorting. For a review of clonal selection and cell separation techniques, see Freshney, Culture of Animal Cells. A Manual of Basic Techniques, 2d Ed., A.R. Liss, Inc., New York, 1987, Ch. 11 and 12, pp. 137-168.

The isolation of fibroblasts may, for example, be carried out as follows: fresh tissue samples are thoroughly washed and minced in Hanks balanced salt solution (HBSS) in order to remove serum. The minced tissue is incubated from 1-12 hours in a freshly prepared solution of a dissociating enzyme such as trypsin. After such incubation, the dissociated cells are suspended, pelleted by centrifugation and plated onto culture

dishes. Fibroblasts will attach before other cells, therefore, appropriate stromal cells can be selectively isolated and grown. The isolated fibroblasts can then be grown to confluency, lifted from the confluent culture and inoculated onto the three-dimensional support (see, Naughton et al., 1987, J. Med. 18(3&4):219-250). Inoculation of the three-dimensional matrix with a high concentration of stromal cells, e.g., approximately 10^6 to 5×10^7 cells/ml, will result in the establishment of the three-dimensional stromal construct in shorter periods of time.

Again, where the cultured cells are to be used for transplantation or implantation in vivo it is preferable to obtain the stromal cells from the patient's own tissues. However, it is also possible to use allogeneic compatible human cells, without significant rejection reactions following transplantation. The growth of cells in the presence of the three-dimensional stromal support matrix may be further enhanced by adding to the matrix, or coating the matrix support with specific amino acids, proteins, glycoproteins, glycosaminoglycans, a cellular matrix, and/or other materials.

After inoculation of the stromal cells, the three-dimensional matrix should be incubated in an appropriate nutrient medium. Many commercially available media such as DMEM, RPMI 1640, Fisher's Iscove's, McCoy's, and the like may be suitable for use. It is preferable that the three-dimensional stromal matrix be suspended or floated in the medium during the incubation period in order to maximize proliferative activity. The container in this protocol is kept stable in the incubator, i.e., under static conditions (no circulating or flowing fluid). In addition, the culture should be "fed" periodically to remove the spent media, depopulate released cells, and add fresh nutrients. The concentration of fibroblasts may be adjusted during these steps. These procedures are greatly facilitated when carried out using a bioreactor,

which is a closed system housing the three-dimensional framework inoculated with stromal cells. A bioreactor reduces the possibility of contamination, maintains the cultures in recirculating, continuous culture medium and keeps the leaflets in a dynamic state by opening and closing them. More particularly, the U.S. patent application entitled "Apparatus and Method for Sterilizing, Seeding, Culturing, Storing, Shipping, and Testing Tissue, Synthetic or Mechanical Heart Valves or Valve Segments" and filed concurrently herewith by the assignee of the present application, teaches the mode of operation of the bioreactor and, is incorporated by reference herein.

During the incubation period, the stromal cells will attach and proliferate along the three-dimensional framework before beginning to migrate into the depths of the matrix. One objective is to grow the cells to an appropriate degree which reflects the amount of stromal cells present in the in vivo tissue. A second objective is to regulate the number of cells in the inoculum and/or their growth on the scaffold such that the amount of scaffold colonization can be controlled as desired, and reproducibly.

The openings of the non-tissue framework or constructs should be of an appropriate size to allow the stromal cells to stretch across the openings. Maintaining actively growing stromal cells which stretch across the framework enhances the production of growth factors which are elaborated by the stromal cells, and hence will support long term cultures. For example, if the openings are too small, the stromal cells may rapidly achieve confluence but be unable to easily exit from the mesh; trapped cells may exhibit contact inhibition and cease production of the appropriate factors necessary to support proliferation and maintain long term cultures. If the openings are too large, the stromal cells may be unable to stretch across the opening; this will also

decrease stromal cell production of the appropriate factors necessary to support proliferation and maintain long term cultures. When using a mesh type of matrix, as exemplified herein, we have found that openings ranging from about 150 μm to about 220 μm will work satisfactory. However, depending upon the three-dimensional structure and intricacy of the framework, other sizes may work equally well. In fact, any shape or structure that allows the stromal cells to stretch and continue to replicate and grow for lengthy time periods will work in accordance with the invention.

The human dermal fibroblasts exhibit a varied affinity for the different types of porcine tissue matrices. The greatest fibroblast colonization occurs when using a porcine matrix that is detergent and/or enzyme extracted. Additionally, the amount of fibroblast colonization in the porcine tissue correlates with time.

Different proportions of the various types of collagen deposited on the framework can be manipulated. For example, for optimal growth of transplantable or bioprosthetic heart valves, collagen types I and III are preferably deposited in the initial matrix. The proportions of collagen types deposited can be manipulated or enhanced by selecting fibroblasts or cells which elaborate the appropriate collagen type. This can be accomplished using monoclonal antibodies of appropriate isotypes or subclass that are capable of activating complement, and which define particular collagen type. These antibodies and complement can be used to negatively select the fibroblasts which express the desired collagen type. Alternatively, the stroma used to inoculate the matrix can be a mixture of cells which synthesize the appropriate collagen types desired. The distribution and origins of the five types of collagen is shown in Table I. Thus, for the growth and preparation of heart valves, fibroblasts are the preferred cells for the present invention.

TABLE I

DISTRIBUTIONS AND ORIGINS OF THE FIVE TYPES OF COLLAGEN

	Tissue Distribution	Cells of Origin
I	connective tissue; collagen fibers	reticular cells; smooth muscle cells
	Fibrocartilage	
	Bone	Osteoblast
	Heart Valve	Fibroblasts
	Dentin	Odontoblasts
II	Hyaline and elastic cartilage	Chondrocytes
	Vitreous body of eye	Retinal cells
III	Loose connective tissue; reticular fibers	Fibroblasts and reticular cells
	Papillary layer of dermis	
	Blood vessels	Smooth muscle cells; endothelial cells
IV	Basement membranes	Epithelial and endothelial cells
	Lens capsule of eye	Lens fibers
V	Fetal membranes; placenta	Fibroblast
	Basement membranes	

Bone	
Smooth muscle	Smooth muscle cells

During incubation of the three-dimensional stromal construct, proliferating cells may be released from the framework. These released cells may stick to the walls of the culture vessel where they may continue to proliferate and form a confluent monolayer. This should be prevented or minimized, for example, by removal of the released cells during feeding, by coating the culture vessel with substances such as silicone to decrease cellular attachment, or by transferring the three-dimensional stromal framework to a new culture vessel. The presence of a confluent monolayer in the vessel will "shut down" the growth of cells in the three-dimensional framework and/or culture. Removal of the confluent monolayer or transfer of the framework to fresh media in a new vessel will restore proliferative activity of the three-dimensional culture system. Such removal or transfers should be done in any culture vessel which has a stromal monolayer exceeding 25% confluency.

Alternatively, the culture system could be agitated to prevent the released cells from adhering, or instead of periodically feeding the cultures, the culture system could be set up so that fresh media continuously flows through the system. The flow rate could be adjusted to both maximize proliferation within the three-dimensional culture, and to wash out and remove cells released from the matrix, so that they will not adhere to the walls of the vessel and grow to confluence.

5.3. USES OF THE TRANSPLANTABLE HUMAN CELL-COLONIZED HEART VALVES AND SHEETS GROWN IN THREE-DIMENSIONAL CULTURE SYSTEM

The three-dimensional culture system of the invention can be used in a variety of applications. These include but are not limited to transplantation or implantation of either the cultured tissue obtained from the framework, or the cultured matrix itself *in vivo*; screening the effectiveness and cytotoxicity of pharmaceutical agents, blood related natural and modified compounds, growth/regulatory factors, etc., *in vitro*; elucidating the mechanism of certain diseases; studying the mechanism by which drugs and/or growth factors operate; gene therapy; and the production of biologically active products, to name but a few.

5.3.1. TRANSPLANTATION IN VIVO

The biological heart valves produced in the three-dimensional culture system of the invention can be used in the treatment of aortic stenosis, aortic regurgitation, mitral stenosis, mitral regurgitation, pulmonary valve disease, tricuspid valve disease, multivalvular disease, tricuspid valve disease, Marfan syndrome and artificial valve disease.

Aortic stenosis is the obstruction to flow across the aortic valve during left ventricular systolic ejection. It can be caused by a congenital unicuspid or bicuspid valve, rheumatic fever, or degenerative calcification of the valve in the elderly. The incidence of bicuspid aortic valve has been estimated at 4 in 1,000 live births, with males dominating over females at 4:1. Campbell, M., and Kauntze, R., 1953, Br. Heart J. 15:179. Leaflets often thicken by age 40 and almost invariably by age 50, but calcium deposits are rarely detected before 40 years of age. Although symptoms generally occur late in the course of aortic stenosis, 3 to 5 percent of patients may die suddenly during an otherwise a

symptomatic period. Thus, patients with any sign of congestive heart failure, angina, or exertional syncope in the presence of significant aortic valvular stenosis should undergo aortic valve replacement promptly. In addition, asymptomatic patients with significant aortic valvular stenosis should be advised to have valve replacement therapy.

Aortic regurgitation is the diastolic flow of blood from the aorta into the left ventricle. It is caused by incompetent closure of the aortic valve which results from intrinsic disease of the cusp or from diseases affecting the aorta. Acquired intrinsic diseases of the aortic valve are either rheumatic or from bacterial origin. In the Marfan syndrome the primary basis for aortic insufficiency usually resides in the aorta, but there may be prolapse of the aortic cusps due to myxomatous changes. Infrequent changes are seen with rheumatoid arthritis, systemic lupus erythematosus, and trauma. Oh, W.M.C., Taylor, T.R. and Olsen, E.G.J., 1974, Br. Heart J. 36:413. Patients with chronic aortic regurgitation who are symptomatic are advised to have surgery. The type of operation used depends primarily on the etiology. In patients with diseases limited to the valve, the operation is essentially as described above, for aortic stenosis.

Mitral stenosis designates resistance to flow through the mitral apparatus during diastolic filling of the left ventricle. Resistance to diastolic flow across the mitral valve can result from rheumatic valvulitis, congenital stenosis, thrombus formation, atrial myxoma, bacterial vegetations, and calcification in the valve, as well as in the annulus. The decision to intervene surgically in patients with mitral stenosis is based on the anticipated necessity of valve replacement versus valve reconstruction therapy.

Mitral regurgitation occurs when contraction of the left ventricle ejects blood into left atrium as a result

of abnormalities in the mitral valve apparatus. Acute mitral regurgitation can be created from mechanical disruption of the chordae tendineae, rupture of the papillary muscle, or perforation of the leaflet. Rheumatic fever, mitral valve prolapse and coronary artery disease, such as left ventricular dilation, calcified mitral annulus, heritable disorders (Marfan syndrome, Ehlers-Danlos, osteogenesis), congenital heart disease, systemic lupus erythematosus, rupture of papillary muscle and perforation of leaflet, are the predominant mechanisms for the incompetence of the mitral valve. Replacement of the mitral valve, valve components and/or other affected parts such as the chordae, is required in cases of rheumatic involvement leading to severe mitral regurgitation, mitral stenosis with loss of pliability of the leaflets, and various other causes of mitral regurgitation, such as infective endocarditis, and in some cases in chronic heart disease. Calcification and immobility of the leaflets are also indications for valve replacement.

Pulmonary stenosis is created by obstruction to systolic flow across the valve and is most commonly congenital. It generally leads to pulmonary regurgitation. Pulmonary valve replacement may be performed for acquired conditions such as carcinoid heart disease and infective endocarditis.

Tricuspid regurgitation develops when the tricuspid valve allows blood to enter the right atrium during right ventricular contraction. Tricuspid stenosis represents obstruction to diastolic flow across the valve during diastolic filling of the right ventricle. The main cause of tricuspid and mitral regurgitation is the rupture of one or more of the elements of the tensor apparatus, with disruption of the papillary muscle and rupture of the chordae tendineae. Replacement is necessary if the changes in the leaflets and subvalvular mechanism are

advanced, or if severe regurgitation cannot be relieved by annulopolasty.

Multivalvular disease indicates obstruction and/or incompetence of the aortic, mitral, and tricuspid valves. Rheumatic fever, connective tissue diseases, Marfan syndrome, calcification of the mitral valve in the aging patient and bacterial endocarditis remain important causes in combined disease of the mitral and aortic valves. In patients with severe and progressive symptoms having evidence of disease at both the mitral and aortic valves, both valves are generally repressed by surgery.

Artificial valve disease includes any abnormality of a surgically implanted device to replace a diseased cardiac valve. Artificial valve disease can result from prosthetic dysfunction, thrombus formation, infection, fibrosis, or calcification. Roberts, W.C., 1973, Prog. Cardiovasc. Dis. 15:539. Congestive heart failure due to mechanical valve dysfunction is the major indication for replacement of a mechanical artificial valve. Replacement of the prosthesis is indicated if the symptoms cannot be controlled medically or if there is evidence of progressive ventricular dysfunction.

The second most common operation performed in adults is replacement of the aortic or mitral valve. The valves produced in accordance with the invention may be transplanted using similar, if not the same surgical techniques, well known to those skilled in the art. The procedure for the replacement of the aortic valve is performed through a median sternum-splitting incision. After cardiopulmonary bypass is begun, a vascular clamp is placed across the distal ascending aorta. A sump suction cannula is placed in the left atrium through an incision in the right superior pulmonary vein to decompress the left heart. A transverse incision is made in the proximal aorta and the diseased valve is excised. Horizontal mattress sutures are placed at the three commissures for traction. Simple radial sutures are then

placed along the annulus between traction sutures and passed sequentially through the sewing ring of the valve as they are inserted. When all sutures have been passed through the sewing ring, the valve is lowered into position and the sutures are tied and cut. The aortotomy is closed with continuous sutures.

Coronary artery perfusion usually is not necessary for single-valve replacement, provided it can be accomplished in 60 minutes or less. Adequate myocardial protection can be afforded by systemic hypothermia at 30·C, injection of cardioplegic solution into the ascending aorta, and lavage of the heart by iced isotonic solution before it is opened.

In case of a mitral valve replacement, tricuspid valve replacement or a pulmonary valve replacement, the procedure is modified accordingly and involves the same technical maneuvers as outlined above. A detailed description of the operative surgery used is described in P.F. Nora, ed., Operative Surgery Principles and Techniques, 2nd ed. (1980) 326-327; J.W. Kirklin and B.G. Barratt-Boyes, eds., Cardiac Surgery Morphology, Diagnostic Criteria, Natural History, Techniques, Results, and Indications, 2d ed. (1993) 498-507; and J.W. Hurst and R.C. Schlant, eds., The Heart, Arteries and Veins, 7th ed. (1990) 795-876.

For transplantation or implantation in vivo, either portions of the culture or the entire three-dimensional culture could be implanted, depending upon the type of tissue involved. For example, three-dimensional heart valve cultures can be maintained in vitro for long periods. Section of tissues or the entire three-dimensional tissue structure can be transplanted in vivo in patients needing new heart valves.

Three-dimensional tissue culture implants may, according to the inventions, be used to replace or augment existing tissue, to introduce new or altered

tissue, to modify artificial prostheses, or to join together biological tissues or structures.

5.3.2. SCREENING EFFECTIVENESS AND CYTOTOXICITY OF COMPOUNDS IN VITRO

The three-dimensional cultures may be used in vitro to screen a wide variety of compounds, for effectiveness and cytotoxicity of pharmaceutical agents, growth/regulatory factors, natural and modified blood products, anticoagulants, clotting agents or anti-calcification agents, etc. To this end, the cultures are maintained in vitro and exposed to the compound to be tested. The activity of a cytotoxic compound can be measured by its ability to damage or kill cells in culture. This may readily be assessed by vital staining techniques. The effect of growth/regulatory factors may be assessed by analyzing the cellular content of the matrix, e.g., by total cell counts, and differential cell counts. This may be accomplished using standard cytological and/or histological techniques including the use of immunocytochemical techniques employing antibodies that define type-specific cellular antigens. The effect of various drugs on normal cells cultured in the three-dimensional system may be assessed.

5.3.3. GENE THERAPY

The three-dimensional culture system of the invention may afford a vehicle for introducing genes and gene products in vivo to assist or improve the results of the transplantation and/or for use in gene therapies. For example, the stromal cells can be genetically engineered to express anticoagulation gene products to reduce the risk of thromboembolism, or anti-inflammatory gene products to reduce the risk of failure due to inflammatory reactions. In this regard, the stromal cells can be genetically engineered to express TPA, streptokinase or urokinase to reduce the risk of

clotting. Alternatively, the stromal cells can be engineered to express anti-inflammatory gene products, for example, peptides or polypeptides corresponding to the idiotype of neutralizing antibodies for TNF, IL-2, or other inflammatory cytokines. Preferably, the cells are engineered to express such gene products transiently and/or under inducible control during the post-operative recovery period, or as a chimeric fusion protein anchored to the stromal cells, for example, a chimeric molecule composed of an intracellular and/or transmembrane domain of a receptor or receptor-like molecule, fused to the gene product as the extracellular domain. In another embodiment, the stromal cells could be genetically engineered to express a gene for which a patient is deficient, or which would exert a therapeutic effect, e.g., HDL, apolipoprotein E, etc. The genes of interest engineered into the stromal cells need to be related to heart disease. For example, the stromal cells can be engineered to express gene products that are carried by the blood; e.g., cerebredase, adenosine deaminase, α-1-antitrypsin. In a particular embodiment, a genetically engineered valve culture implanted to replace the pulmonary valve can be used to deliver gene products such as α-1-antitrypsin to the lungs; in such an approach, constitutive expression of the gene product is preferred.

The stromal cells can be engineered using a recombinant DNA construct containing the gene used to transform or transfect a host cell which is cloned and then clonally expanded in the three-dimensional culture system. The three-dimensional culture which expresses the active gene product, could be implanted into an individual who is deficient for that product. For example, genes that prevent or ameliorate symptoms of various types of valvular heart diseases may be underexpressed or down regulated under disease conditions. Specifically, expression of genes involved in preventing the following pathological conditions may

be down-regulated, for example: thrombus formation, inflammatory reactions, and fibrosis and calcification of the valves. Alternatively, the activity of gene products may be diminished, leading to the manifestations of some or all of the above pathological conditions and eventual development of symptoms of valvular disease. Thus, the level of gene activity may be increased by either increasing the level of gene product present or by increasing the level of the active gene product which is present in the three-dimensional culture system. The three-dimensional culture which expresses the active target gene product can then be implanted into the valvular disease patient who is deficient for that product. "Target gene," as used herein, refers to a gene involved in valvular disease in a manner by which modulation of the level of target gene expression or of target gene product activity may act to ameliorate symptoms of valvular disease.

Further, patients may be treated by gene replacement therapy during the post-recovery period after transplantation. Heart valve constructs or sheets may be designed specifically to meet the requirements of an individual patient, for example, the stromal cells may be genetically engineered to regulate one or more genes; or the regulation of gene expression may be transient or long-term; or the gene activity may be non-inducible or inducible. For example, one or more copies of a normal target gene, or a portion of the gene that directs the production of a normal target gene protein product with target gene function, may be inserted into human cells that populate the three-dimensional constructs using either non-inducible vectors including, but are not limited to, adenovirus, adeno-associated virus, and retrovirus vectors, or inducible promoters, including metallothionein, or heat shock protein, in addition to other particles that introduce DNA into cells, such as liposomes or direct DNA injection or in gold particles.

For example, the gene encoding the human complement regulatory protein, which prevents rejection of the graft by the host, may be inserted into human fibroblasts. Nature 375: 89 (May, 1995).

The three-dimensional cultures containing such genetically engineered stromal cells, e.g., either mixtures of stromal cells each expressing a different desired gene product, or a stromal cell engineered to express several specific genes are then implanted into the patient to allow for the amelioration of the symptoms of valvular disease. The gene expression may be under the control of a non-inducible (i.e., constitutive) or inducible promoter. The level of gene expression and the type of gene regulated can be controlled depending upon the treatment modality being followed for an individual patient.

The use of the three-dimensional culture in gene therapy has a number of advantages. Firstly, since the culture comprises eukaryotic cells, the gene product will be properly expressed and processed in culture to form an active product. Secondly, gene therapy techniques are useful only if the number of transfected cells can be substantially enhanced to be of clinical value, relevance, and utility; the three-dimensional cultures of the invention allow for expansion of the number of transfected cells and amplification (via cell division) of transfected cells.

A variety of methods may be used to obtain the constitutive or transient expression of gene products engineered into the stromal cells. For example, the transkaryotic implantation technique described by Seldon, R.F., et al., 1987, Science 236:714-718 can be used. "Transkaryotic", as used herein, suggests that the nuclei of the implanted cells have been altered by the addition of DNA sequences by stable or transient transfection. The cells can be engineered using any of the variety of vectors including, but not limited to, intergating viral

vectors, e.g., retrovirus vector or adeno-associated viral vectors, or non-integrating replicating vectors, e.g., papilloma virus vectors, SV40 vectors, adenoviral vectors; or replication-defective viral vectors. Where transient expression is desired, non-integrating vectors and replication defective vectors may be preferred, since either inducible or constitutive promoters can be used in these systems to control expression of the gene of interest. Alternatively, integrating vectors can be used to obtain transient expression, provided the gene of interest is controlled by an inducible promoter.

Preferably, the expression control elements used should allow for the regulated expression of the gene so that the product is synthesized only when needed in vivo. The promoter chosen would depend, in part upon the type of tissue and cells cultured. Cells and tissues which are capable of secreting proteins (e.g., those characterized by abundant rough endoplasmic reticulum, and golgi complex) are preferable. Hosts cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.) and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which, in turn, can be cloned and expanded into cell lines. This method can advantageously be used to engineer cell lines which express the gene protein product.

Any promoter may be used to drive the expression of the inserted gene. For example, viral promoters include but are not limited to the CMV promoter/enhancer, SV 40, papillomavirus, Epstein-Barr virus, elastin gene promoter and β-globin. If transient expression is desired, such

constitutive promoters are preferably used in a non-integrating and/or replication-defective vector. Alternatively, inducible promoters could be used to drive the expression of the inserted gene when necessary. For example, inducible promoters include, but are not limited to, metallothionein and heat shock protein.

Examples of transcriptional control regions that exhibit tissue specificity for connective tissues which have been described and could be used, include but are not limited to: elastin or elastase I gene control region which is active in pancreatic acinar cells (Swit et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515). The deposition of elastin is correlated with specific physiological and developmental events in different tissues, including the heart valves. For example, atrioventricular valve cusps are initially thick and fleshy in an embryo, and later in the development are transformed into thin and fibrous cusps. In developing arteries, elastin deposition appears to be coordinated with changes in arterial pressure and mechanical activity. Animals that contain valves and ligamental structures that are elastic contain elastin. The transduction mechanisms that link mechanical activity to elastin expression involve cell-surface receptors. Once elastin-synthesizing cells are attached to elastin through cell-surface receptors, the synthesis of additional elastin and other matrix proteins may be influenced by exposure to stress or mechanical forces in the tissue (for example, the constant movement of the construct in the bioreactor) or other factors that influence cellular shape.

Once genetically engineered cells are implanted into an individual, the presence of TPA, streptokinase or urokinase activity can bring about amelioration of platelet aggregation, blood coagulation or thromboembolism. This activity is maintained for a

limited time only, for example, to prevent potential complications that generally develop during the early phase after valve implantation, such as, platelet aggregation, blood clotting, coagulation or thromboembolism. Alternatively, once genetically engineered cells are implanted into an individual, the presence of the anti-inflammatory gene products, for example, peptides or polypeptides corresponding to the idiotype of neutralizing antibodies for TNF, IL-2, or other inflammatory cytokines, can bring about amelioration of the inflammatory reactions associated with valvular disease.

The stromal cells used in the three-dimensional culture system of the invention may be genetically engineered to "knock out" expression of factors or surface antigens that promote clotting or rejection at the implant site. Negative modulatory techniques for the reduction of target gene expression levels or target gene product activity levels are discussed below. "Negative modulation", as used herein, refers to a reduction in the level and/or activity of target gene product relative to the level and/or activity of the target gene product in the absence of the modulatory treatment. The expression of a gene native to stromal cell can be reduced or knocked out using a number of techniques, for example, expression may be inhibited by inactivating the gene completely (commonly termed "knockout") using the homologous recombination technique. Usually, an exon encoding an important region of the protein (or an exon 5' to that region) is interrupted by a positive selectable marker (for example *neo*), preventing the production of normal mRNA from the target gene and resulting in inactivation of the gene. A gene may also be inactivated by creating a deletion in part of a gene, or by deleting the entire gene. By using a construct with two regions of homology to the target gene that are far apart in the genome, the sequences intervening the

two regions can be deleted. Mombaerts, P., et al., 1991, Proc. Nat. Acad. Sci. U.S.A. 88:3084-3087.

Antisense and ribozyme molecules which inhibit expression of the target gene can also be used in accordance with the invention to reduce the level of target gene activity. For example, antisense RNA molecules which inhibit the expression of major histocompatibility gene complexes (HLA) shown to be most versatile with respect to immune responses. Still further, triple helix molecules can be utilized in reducing the level of target gene activity. These techniques are described in detail by L.G. Davis, et al., eds, Basic Methods in Molecular Biology, 2nd ed., Appleton & Lange, Norwalk, Conn. 1994.

Using any of the foregoing techniques, the expression of fibrinogen, von Willebrands factor, factor V or any cell surface molecule that binds to the platelet $\alpha 2B\beta$-3 receptor can be knocked out in the stromal cells to reduce the risk of clot formation at the valve. Likewise, the expression of MHC class II molecules can be knocked out in order to reduce the risk of rejection of the graft.

In yet another embodiment of the invention, the three-dimensional culture system could be used in vitro to produce biological products in high yield. For example, a cell which naturally produces large quantities of a particular biological product (e.g., a growth factor, regulatory factor, peptide hormone, antibody, etc.), or a host cell genetically engineered to produce a foreign gene product, could be clonally expanded using the three-dimensional culture system in vitro. If the transformed cell excretes the gene product into the nutrient medium, the product may be readily isolated from the spent or conditioned medium using standard separation techniques (e.g., HPLC, column chromatography, electrophoretic techniques, to name but a few). A "bioreactor" has been devised which takes advantage of

the flow method for feeding the three-dimensional cultures in vitro. Essentially, as fresh media is passed through the three-dimensional culture, the gene product is washed out of the culture along with the cells released from the culture. The gene product is isolated (e.g., by HPLC column chromatography, electrophoresis, etc.) from the outflow of spent or conditioned media.

6. EXAMPLE: THREE-DIMENSIONAL HEART VALVE CULTURE SYSTEM

The three-dimensional culture of the present invention provides for the growth of stromal cells such as fibroblasts upon decellularized heart valves in vitro, in a system designed to mimic physiologic conditions in vivo. Importantly, the cells replicated in this system synthesize proteins similar to those produced by the normal aortic wall and leaflet cells.

Heart valve extracellular matrix is composed mainly of elastin and collagen types I and III. The following example describes a method for growing transplantable or bioprosthetic heart valve tissue in culture by inoculating stromal cells from an exogenous source on aortic walls and leaflets, and obtaining morphologically and functionally normal human cells proliferating on the three-dimensional framework.

6.1. MATERIAL AND METHODS

6.1.1. CELLS AND PORCINE TISSUE

Porcine aortic walls and leaflets were washed with phosphate buffered saline and used fresh or after being frozen at -20°C to -70°C in sterile water or after detergent and/or enzyme extraction or any aforementioned tissue in combination with sterilization techniques as described in U.S. Patent No. 4,776,85. See Section 5.1.

Dermal foreskin fibroblasts were cultured in vitro by routine procedures. Fibroblasts used in the studies

were in their eighth passage at the time of seeding to the porcine tissues.

6.1.2. FOUR-WEEK IN VITRO CULTURES OF PORCINE AORTIC LEAFLETS AND WALLS

Porcine aortic leaflets and walls were seeded in eight well dishes with $1x10^5$ human dermal fibroblasts and cultured for one day. The aortic walls and leaflets were transferred into new well dishes and grown for an additional four weeks. The eight cultures were made up of: (1) previously frozen leaflet seeded with human fibroblasts; (2) previously frozen wall seeded with human fibroblasts; (3) previously frozen leaflet without seeding; (4) previously frozen wall without seeding; (5) fresh leaflet seeded with human fibroblasts; (6) fresh wall seeded with human fibroblasts; (7) fresh leaflet without seeding; and (8) fresh wall without seeding. The cultures were labeled with [^{35}S]-methionine and [^{35}S]-cysteine (Tran ^{35}S-Label, ICN) for four hours. The samples were boiled in Laemmli sample buffer containing β-mercaptoethanol, fractionated by SDS polyacrylamide gel electrophoresis (SDS•PAGE), and analyzed by autoradiography.

6.1.3. LESS THAN 1 WEEK TO 18-WEEK IN VITRO CULTURES OF PORCINE AORTIC LEAFLETS AND WALLS

Decellularized porcine leaflet or wall tissues were housed in a multi-well dish (one piece of tissue/well) as described above in Section 6.1.2. The human dermal fibroblasts were suspended in a nutrient-rich growth medium and were seeded onto the specific types of porcine leaflet or wall tissues such as: 1) frozen leaflets and walls; 2) electron beamed leaflets; 3) detergent and/or enzyme extracted leaflets and walls; and 4) detergent and/or enzyme extracted + electron beamed leaflets.

Each culture dish was maintained at 37°C in a sterile, static culture (no media flow) environment.

These human fibroblast-porcine tissue composites which grow in the tissue culture dish are referred to as heart valve constructs. The constructs were analyzed for:

a) Cell Distribution

In order to track the distribution and migration of the human dermal fibroblasts into the porcine tissue matrix, an antibody (human anti-prolyl-4-hydroxylase) (DAKO-O-Fibroblast, 5B5, Code No. M 877) was used to identify the cells post-colonization. The antibody does not cross-react with porcine tissue. See Figure 5.

b) Cell Viability

i) MTT Assay: This assay is used to assess the viability of cells after growing on the porcine matrix. Metabolically active (living) fibroblasts convert the MTT substrate (0.5mg/ml) into an insoluble purple precipitant within the cells. The purple precipitant can be visualized by the naked eye and this reflects the pattern of the viable fibroblast distribution on the porcine matrix. The MTT reaction can be quantified by measuring the optical density with a spectrophotometer (540nm) after extraction in isopropanol as described in Triglia, D., et al., 1991, Toxic. in Vitro 5:573-578.

ii) Glucose consumption: As an indicator of fibroblast viability, nutrient consumption (glucose) and metabolic waste products (lactate) contained in the tissue construct are measured as described in Halberstadt, C.R., et al., 1994, Biotechnology and Bioengineering 43:740-746. Viable fibroblasts decrease the concentration of glucose over time and increase the concentration of lactate.

c) Cell Proliferation

i) Thymidine [^{3}H-thy] Incorporation: Radioactive thymidine (10 μCi) is added to the nutrient-rich media during the 24-72 hr culture of tissue constructs. When

fibroblasts in the constructs divide to produce additional cells, some of the ^{3}H-thy becomes incorporated in the DNA of the cells. Excess, non-incorporated ^{3}H-thy is removed after washing the labelled constructs in 1% triton-X-100 for 2 hr and rinsing in PBS. The incorporated ^{3}H-thy can be measured using a scintillation counter.

ii) BrdU Incorporation: An alternative method for measuring fibroblast proliferation in tissue constructs is to add 5-bromodeoxyuridine (BrdU) to the culture media. BrdU is a non-radioactive, thymidine analog which incorporates into newly synthesized DNA of dividing fibroblasts. Tissues are incubated in BrdU-containing media for 24-48 hr. The fibroblasts containing BrdU can be visualized in the histology sections of the tissue constructs using a monoclonal antibody to the BrdU, followed by an enzyme-chromogen detection system using the Zymed Kit. (ZYMED Laboratories, Inc. San Francisco, Ca).

d) Protein Assays

These methods utilize radiolabelled amino acids which are added to the nutrient-rich media during the tissue culture process. The radiolabelled amino acids are incorporated into newly synthesized proteins in the tissue constructs and can be measured using a scintillation counter and/or extracted and separated on a polyacrylamide (10%) gel by their molecular weights. The gel is washed in salicylic acid (1M), then exposed to an X-ray film (4-16 hr at 4 -25°C) which, upon developing, detects the images of radiolabelled proteins.

i) ^{3}H-Proline Labeling: Proline is a major amino acid constituent of the collagen proteins in the tissue constructs. The amount of radioactive proline incorporated (incubation in 10μCi/ml for 24-72 hr) is quantified by scintillation counting to reflect the amount of collagen being newly synthesized. Excess, non-

incorporated label is removed after washing the labeled constructs in 1% triton X-100. Ascorbic acid (25-50μg/ml) can be used as a positive inducer of collagen synthesis through activation of the propyl4-hydroxylase enzyme.

ii) ^{35}S-Cysteine/Methionine Labeling: Constructs are incubated for 0.5 hr in medium free of cysteine and methionine, then in medium containing labeled cysteine and methionine (0.2mCi/ml) for 4 hr. The radioactive isotopes are incorporated into newly synthesized proteins. The labelled tissues can then be digested in laemeli sample buffer under reducing (β-mercaptoethanol) conditions and separated by SDS-PAGE. Specific proteins can be quantified by western blotting.

e) Protein Immunohistochemistry

This method detects specific proteins in a histological section of tissue using monoclonal or polyclonal antibodies. The antibodies used specifically detect human proteins and react with: 1) human fibroblasts (prolyl-4-hydroxylase); 2) a small component of human elastin fibers (in valve wall and leaflet tissue); and 3) human tenascin (matrix glycoprotein). The antibody to the target protein is added to deparaffined or frozen sections. A second antibody which recognizes the primary antibody, is conjugated to an enzyme-chromogen visualization system.

6.1.4. IN VITRO COLONIZATION OF AORTIC WALLS AND LEAFLETS UNDER DYNAMIC CONDITIONS

Porcine leaflets were glued (medical grade cyanoacrylate) either along one surface (immobilizing the tissue) or on one edge (allowing some movement) to a polycarbonate cassette sterilized by electron beam radiation (E-beam). Human fibroblasts were seeded dynamically (5 ml/min flow rate) on these tissues and cultured for three days. The tissues were excised from

the cassette and labeled with [^{35}S]-methionine and [^{35}S]-cysteine for four hours. Tissues were boiled in Laemmli sample buffer, insoluble material was pelleted, and supernatants were fractionated by SDS•PAGE and visualized by autoradiography.

6.1.5. IN VITRO COLONIZATION OF WHOLE AORTIC VALVES UNDER DYNAMIC CONDITIONS

Whole porcine valves were either sterilized by E-beam radiation or disinfected by an antibiotic/antimycotic solution. Valves were placed in E-beam sterilized "bioreactor" and seeded with 40-50 x10^6 cells at a flow rate of 15-50 ml/min in recirculating nutrient-rich medium. After culture for up to 4 (or 8 weeks)with nutrient exchanges every week, the constructs were evaluated by MTT assay, histological staining and immunohistochemistry.

6.2. RESULTS

6.2.1. PORCINE AORTIC LEAFLETS AND WALLS

While the frozen, thawed, unseeded aortic leaflets and walls did not incorporate appreciable amounts of label (Fig. 1, lanes 3 and 4, respectively), the leaflets and walls which were seeded with human fibroblasts incorporated the radioactive amino acid precursors (^{35}S-cys/met) and synthesized proteins ranging in molecular weight from approximately 29,000 to 200,000 daltons (Fig. 1, lanes 1 and 2, respectively).

Lanes 5 through 8 describe corresponding results obtained with fresh, unfrozen leaflets and walls. Seeding fresh, unfrozen aortic leaflets and walls with human fibroblasts, resulted in an increase in the incorporation of the amount of radioactivity, in lanes 5 and 6. This increase is similar to that observed in lanes 1 and 2, respectively. Lanes 7 and 8, containing unseeded fresh, unfrozen aortic leaflets and walls,

respectively, demonstrated protein synthesis by endogenous viable cells. The aortic walls were less metabolically active than the leaflets. Of particular interest is the fact that the protein profile shown in lane 7 is similar to the protein profile in lane 1, indicating that proteins synthesized by fibroblasts seeded onto frozen porcine leaflets are similar to proteins that are synthesized by the endogenous cells of normal, fresh porcine leaflets.

6.2.2. COLONIZATION OF AORTIC WALLS AND LEAFLETS AT 8-18 WEEKS

Human dermal fibroblasts were able to colonize every tissue type from biopsies of aortic leaflets and walls over all time intervals described in Table II. The greatest fibroblast penetration of the porcine matrix occurred in leaflets, specifically in detergent and/or enzyme extracted leaflets (Figure 2). Overall, the cell distribution in the detergent and/or enzyme extracted leaflets cultured 8 to 18 weeks, appeared to approach cell densities typical of a fresh porcine leaflet. Figure 3 represents cell distribution in detergent and/or enzyme extracted leaflets cultured for 4 weeks.

Cell viability assessments (MTT assay) demonstrated that the human fibroblasts remained metabolically alive even after 18 weeks. The fibroblasts were also shown to be proliferating (^{3}H-thy incorporation assay) throughout the culture process (Figure 4).

Protein production, measured as collagen synthesis (^{3}H-proline labeling) indicated that the human dermal fibroblasts were producing collagen and some proteins that are present in porcine leaflets (^{35}S-cysteine/methionine labeling) (Figure 5).

Proteins typical of heart valve tissue were identified by immunohistochemistry using specific antibodies. Fibroblasts produced human tenascin to supplement the existing porcine scaffolding (Figure 6).

6.2.3. COLONIZATION OF AORTIC WALLS AND LEAFLETS UNDER DYNAMIC CONDITIONS

Lanes 1 and 2 (Fig. 7) containing samples of porcine leaflets that were glued along their entire surface, and cultured under dynamic flow had no appreciable staining. Lanes 3 and 4 show porcine leaflets that were glued on one edge, with an appreciable amount of radioactivity was incorporated after growth for three days post seeding; when porcine leaflets were glued along an entire surface, minimal [^{35}S] was incorporated into protein. An unseeded, E-beam sterilized leaflet used as a control (lane 5) showed no incorporation of radioactivity.

Thus, porcine aortic leaflets and walls can be statically or dynamically seeded with human fibroblasts. These human fibroblasts attach and colonize the aortic leaflet and wall scaffolds, and remain metabolically active by secreting extracellular matrix molecules.

6.2.4. IN VITRO COLONIZATION OF WHOLE AORTIC VALVES UNDER DYNAMIC CONDITIONS

Human fibroblasts grew under dynamic conditions, on a porcine matrix which was previously decellularized by detergent and/or enzyme treatment. The proliferating cells were labeled with Brdu and detected using an antibody to Brdu. See Figure 8.

When the human fibroblasts were grown on a porcine matrix which was previously decellularized by detergent and/or enzyme treatment + electron beamed, the matrix seeded under dynamic, pulstile flow conditions had greater and more uniform fibroblast attachment that the matrix grown under static conditions, as shown by using the MTT assay (an indicator of cell viability as described in Section 6.1.3 (b) above). See Figure 9.

The present invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and

components are within the scope of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A living stromal cell-colonized heart valve prepared *in vitro*, comprising stromal cells and connective tissue proteins naturally secreted by the stromal cells which are inoculated upon a heart valve such that the heart valve becomes repopulated with viable cells to form a three-dimensional structure having interstitial spaces bridged by the stromal cells.

2. The living stromal cell-colonized heart valve of Claim 1 in which the stromal cells are fibroblasts.

3. The living stromal cell-colonized heart valve of Claim 1 in which the stromal cells are human dermal fibroblasts.

4. The living stromal cell-colonized heart valve of Claim 1 in which the stromal cells are human cardiac fibroblasts.

5. The living stromal cell-colonized heart valve of Claim 1 in which the heart valve is of porcine origin.

6. The living stromal cell-colonized heart valve of Claim 5 in which the heart valve is decellularized prior to stromal cell inoculation.

7. The living stromal cell-colonized heart valve of Claim 6 in which the heart valve is decellularized by enzymatic and detergent treatment or made non-viable by freezing/thawing.

8. The living stromal cell-colonized heart valve of Claim 1 in which the heart valve is composed of biodegradable material.

9. The living stromal cell-colonized heart valve of Claim 8 in which the biodegradable material comprises polyglycolic acid, catgut sutures, collagen, cellulose, gelatin, hyaluronic acid or polyhydroxyalkanoates.

10. A method for preparing a living stromal cell-coated heart valve *in vitro*, comprising culturing stromal cells inoculated onto a heart valve in a culture medium, so that the stromal cells and connective tissue human matrix proteins naturally secreted by the stromal cells attach to the heart valve to form into a three-dimensional construct.

11. The method of Claim 10 in which the stromal cells are fibroblasts.

12. The method of Claim 10 in which the stromal cells are human dermal fibroblasts.

13. The method of Claim 10 in which the stromal cells are human cardiac fibroblasts.

14. The method of Claim 10 in which the heart valve is of porcine origin.

15. The method of Claim 14 in which the heart valve is decellularized prior to stromal cell inoculation.

16. The method of Claim 15 in which the heart valve is decellularized by enzymatic and detergent treatment or made non-viable by freezing/thawing.

17. The method of Claim 10 in which the heart valve is composed of biodegradable matrices.

18. The method of Claim 17 in which the biodegradable material comprises polyglycolic acid,

catgut sutures, cellulose, collagen, gelatin, hyaluronic acid or polyhydroxyalkanoates.

19. A method of Claim 10 in which the culture medium is kept under static conditions.

20. A method of Claim 10 in which the culture medium is kept in a dynamic state by recirculating the culture medium.

21. A method of Claim 10 further comprising the use of growth factors, natural or modified blood products or bioactive molecules in the culture medium.

22. A method for transplantation or implantation of a living stromal cell-colonized heart value comprising,

(a) inoculating stromal cells on a decellularized heart valve;

(b) culturing the stromal cells so that they proliferate *in vitro* ; and

(c) implanting the stromal cell-coated humanized heart valve construct *in vivo*.

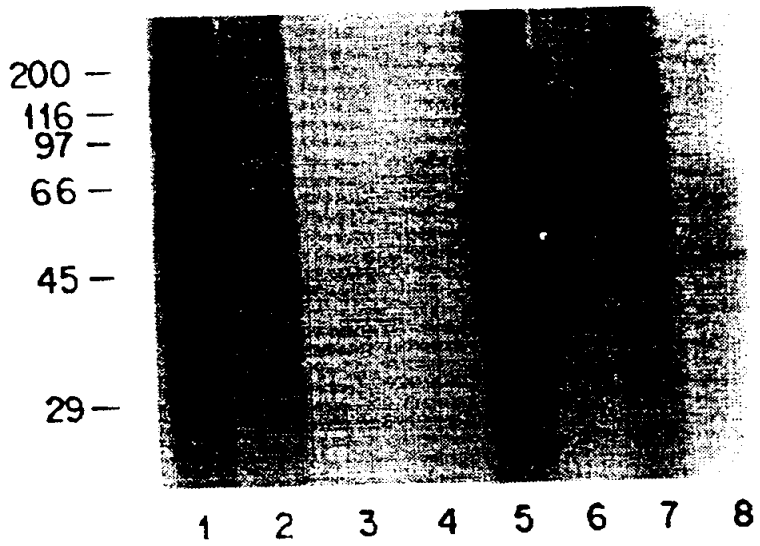

FIG. 1

FIG.2A

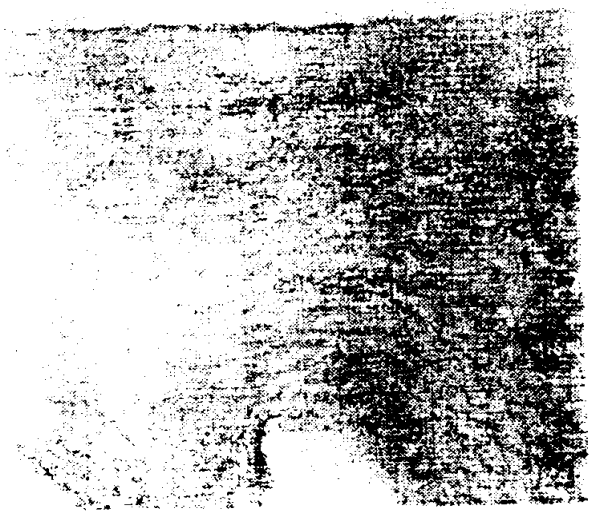

FIG.2B

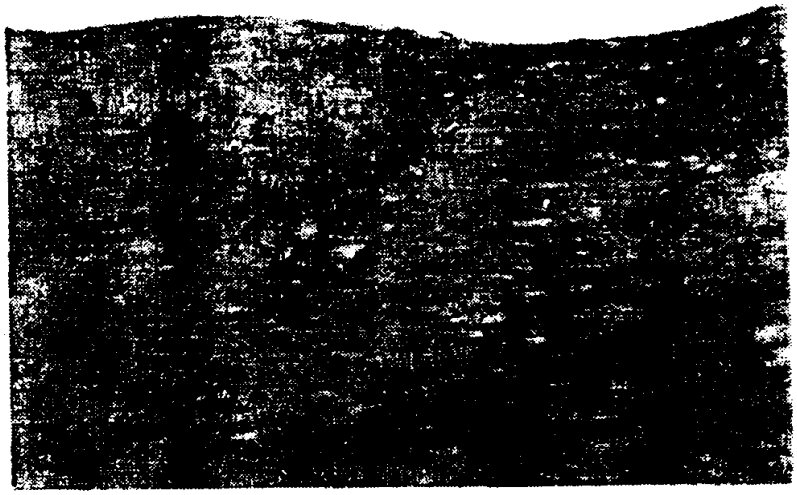

FIG.2C

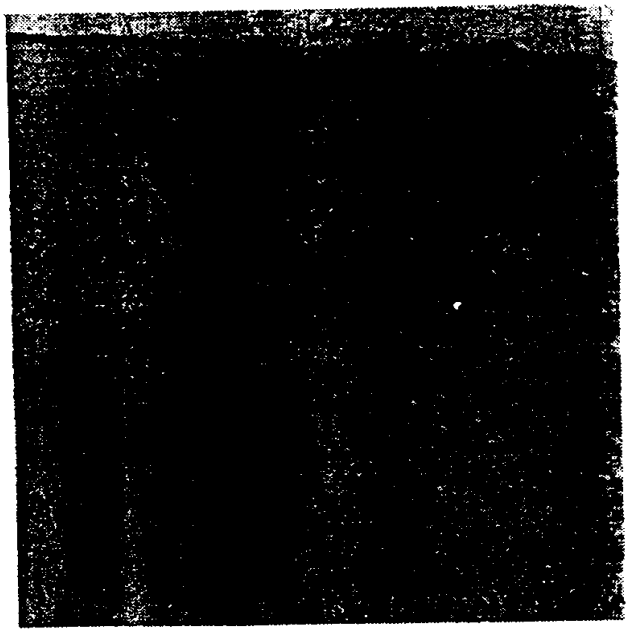

FIG.3

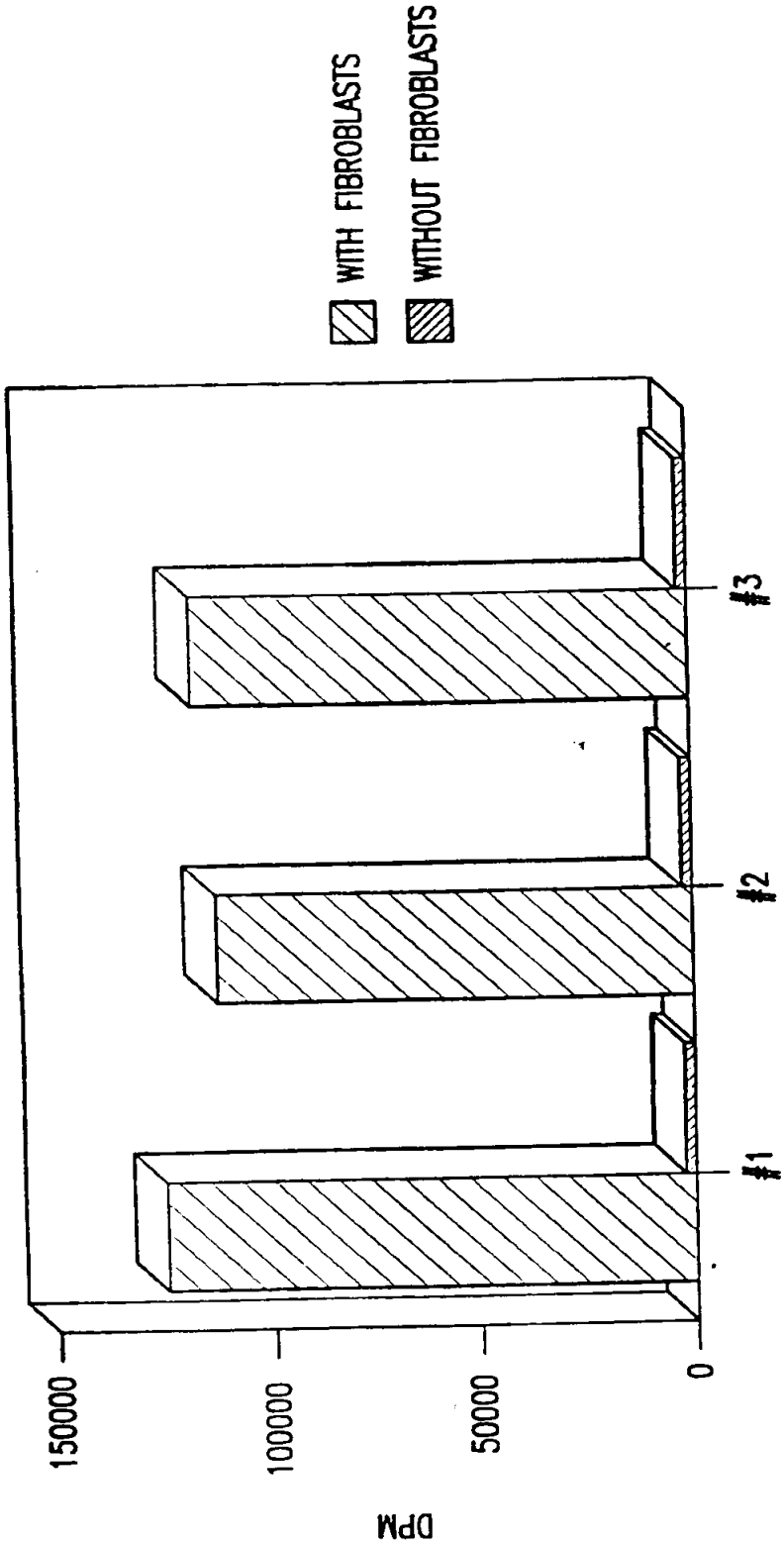
WITH FIBROBLASTS
WITHOUT FIBROBLASTS
#3
#2
#1
150000
100000
50000
0
DPM

FIG.4

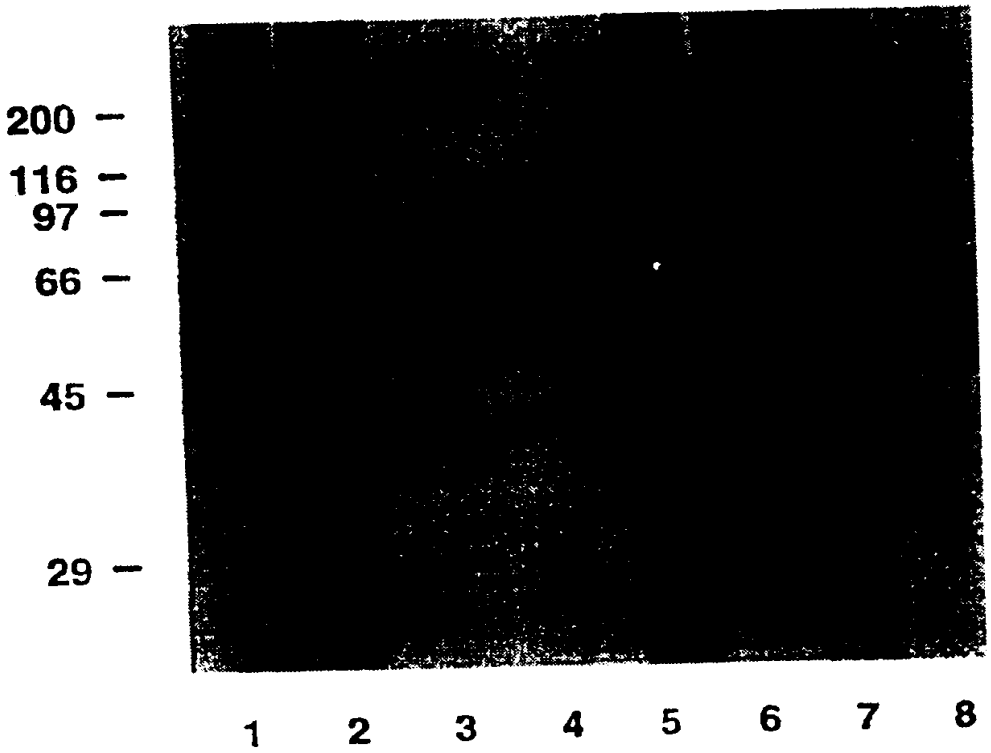

FIG.5

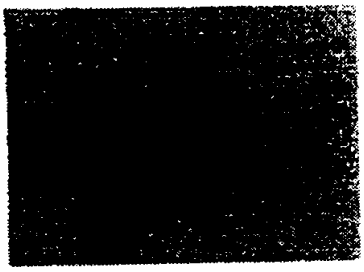

FIG.6A

FIG.6B

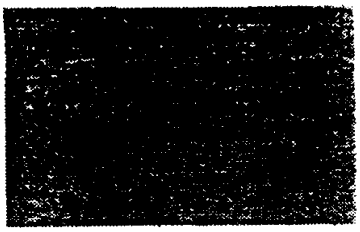

FIG.6C

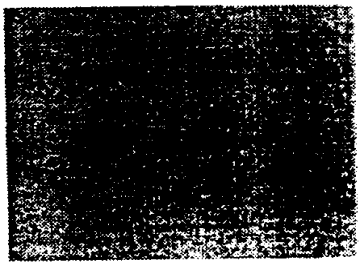

FIG.6D

FIG.6E

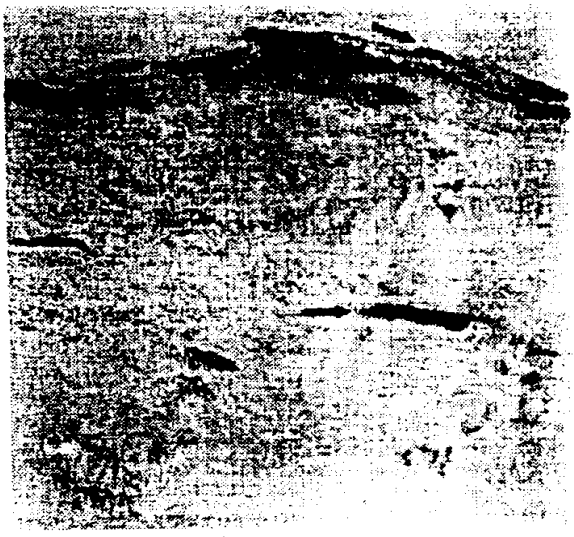

FIG.6F

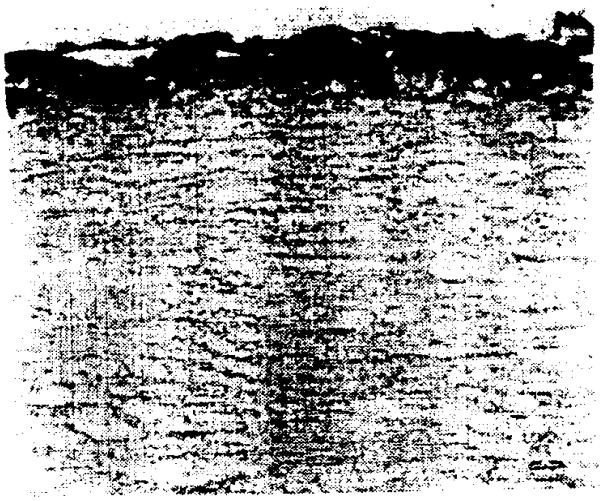

FIG.6G

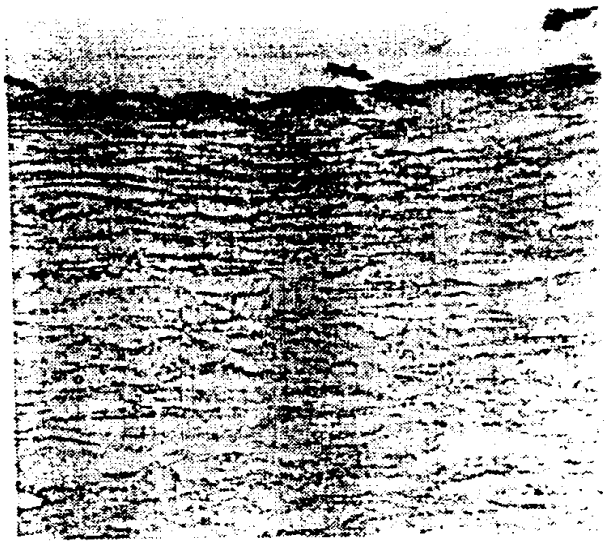

FIG.6H

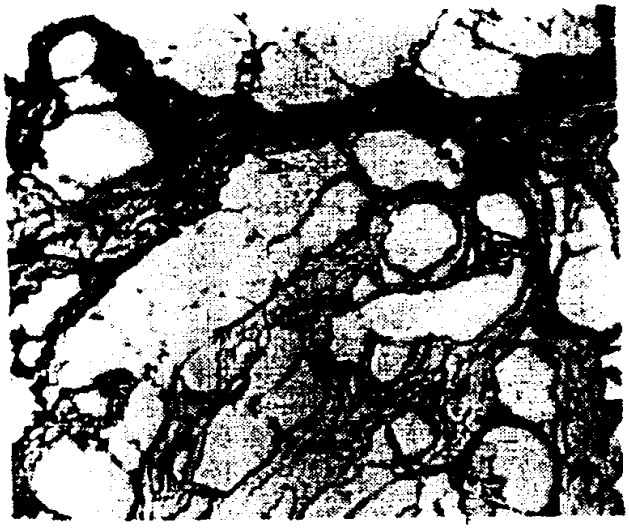

FIG.6I

FIG.6J

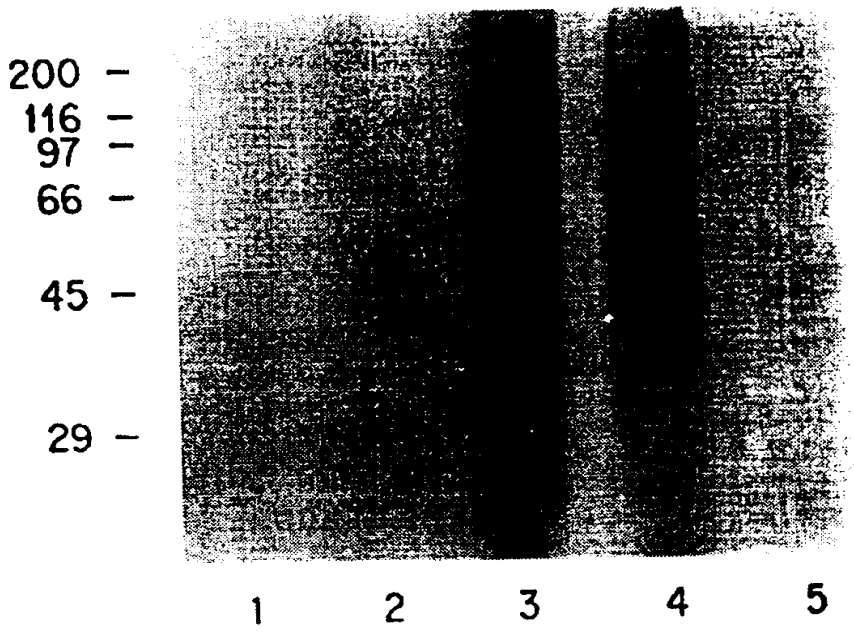

FIG.7

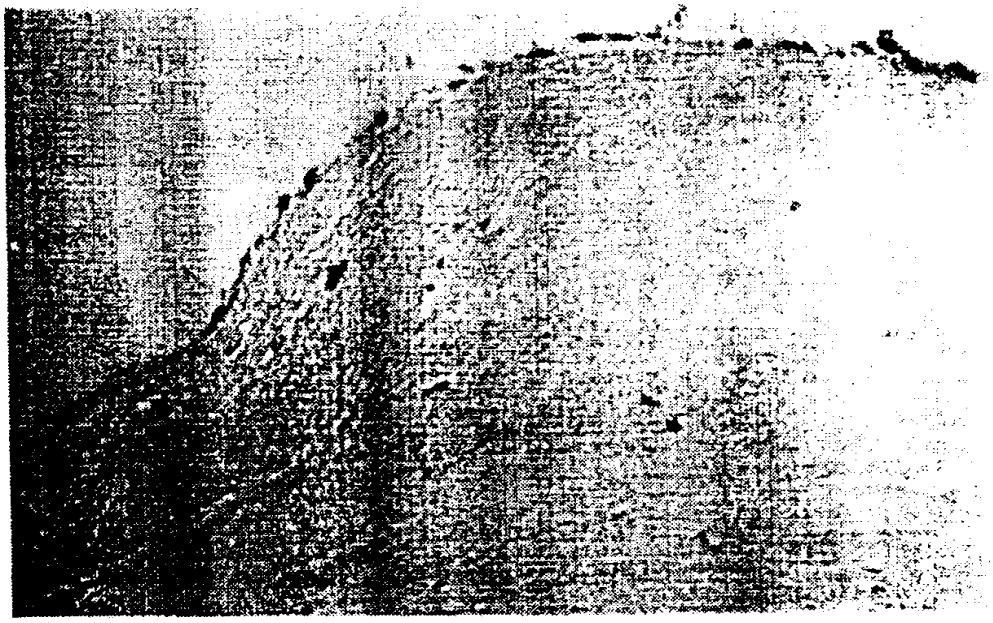

FIG.8

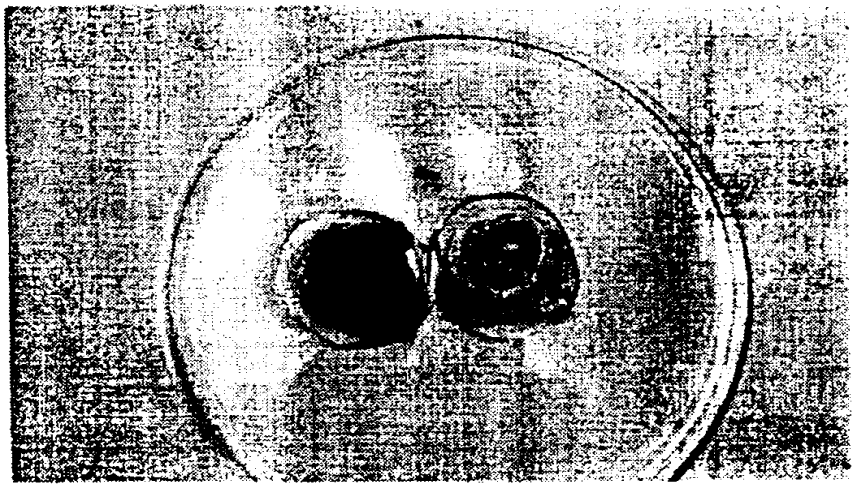

FIG.9

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US95/11395

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61F 2.24
US CL :623/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 623/2, 11

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS search terms: heart valve# and stroma#(10a)cell# and fibroblast# and porcine and decelluliz?.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 5,192,312 (ORTON ET AL) 09 March 1993, see the entire document.	1, 2, 8-11, 17-19, and 21
Y		3-7, 12-16, 20 and 22
Y	US, A, 5,336,616 (LIVESEY ET AL) 09 August 1994, see the entire document.	1-22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
27 OCTOBER 1995

Date of mailing of the international search report
26 DEC 1995

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